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Stability of a 50 mg/mL Ceftazidime Eye-Drops Formulation

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Abstract

Background: Microbial keratitis are severe infections generally linked to risk factors. High-doses antibiotic eye-drops could be required to avoid severe complications. In such cases, hospital pharmacists are in charge of their production given the lack of such formulations on the market. The stability of these antibiotic eye-drops is generally limited to a couple of days and publications generally do not describe addition of microbial preservatives even though it is a European Pharmacopeia requirement. The aim of this study was to describe a new ceftazidime eye-drops formulation at 50 mg/mL with a antimicrobial additive, benzalkonium chloride at 0.04 mg/mL.

Methods: Physico-chemical studies of this new formulation were performed by a stability indicating HPLC-UV method validated according to ICH standards, osmolality measurements, pH monitoring and visual examinations. Antimicrobial preservative efficacy was evaluated according to the method from the European Pharmacopeia.

Results: After 75 days at −20 °C followed by 7 days at 4 °C, or after 7 days at 4 °C, the eye-drops were stable. A degradation trend was finally observed at day 14 at 4 °C.

Conclusions: A new ceftazidime eye-drops formulation is proposed with a stability of 7 days. Outpatients do not need to return to the hospital pharmacy for repeat dispensing, thus possibly improving treatment compliance.

Keywords: ceftazidime, eyedrops, stability, HPLC UV, stability indicating method

Introduction

Microbial keratitis, a suppurative corneal infiltrate associated with bacterial colonization, is a severe infection generally linked to a risk factor, such as contact or cosmetic lenses, ocular disease, eye surgery or immunodepression [1–3].

Bacterial keratitis requires instituting a rapid and effective antibiotic eye-drops regime to avoid severe complications such as visual impairment, corneal opacity, endophthalmitis or in the worst case, visual loss. Treatment, based on high-dose antibiotics, must be started without delay after clinical presentation [4]. Antibiotics used in these circumstances could be amikacin, cefazolin, ceftazidime, gentamycin, ticarcillin or vancomycin. They are used alone or in association (bi or tritherapy), depending on the suspected germ involved in the bacterial keratitis. A corneal scraping should be performed before starting antibiotic treatment. Antibiotics are thus used as a probabilistic treatment before culture data on germ identification and antibiotic susceptibility. Antibiotherapy is then adapted when the results of the microbiology evaluation become available.

Hospital pharmacists are in charge of the production of fortified antibiotics eye-drops corresponding to high-dose antibiotics not commercially available. Eye-drops dispensation is initiated in hospital during patient hospitalization and could be continued for several days or weeks, depending on the progression of the infection [5]. The stability of such antibiotic eye-drops are generally limited to a couple of days [6, 7]. To be produced in advance and be ready to use at any time, they could be frozen at −20 °C or −80 °C to

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prolong stability. However, once thawed, the stability remains very short [7–10], leading to multiple dispensing which is a major drawback for outpatients, with possible treatment interruptions [5].

The aetiology of bacterial keratitis could be different from one geographical region to another depending on economic factors, and rural or urban localization [11]. It is therefore necessary to know the aetiology of the main germs involved in such a pathology in order to be able to offer clinicians effective antibiotics. In Europe, Gram positive bacteria are largely encountered with a majority of *Staphylococci*, followed by Gram negative bacteria mainly represented by *Pseudomonas aeruginosa* [5, 11, 12]. In this case ceftazidime could be used due to the sensitivity of *Pseudomonas aeruginosa* [12, 13].

Ceftazidime is a β-lactam antibiotic, more specifically a third-generation cephalosporin. It is widely used to treat severe infections caused by Gram negative bacilli, including *Pseudomonas aeruginosa*. This bactericidal antibiotic is available for intravenous or intramuscular injections only. Ceftazidime eye-drops are not commercially available due to a rapid degradation of ceftazidime in aqueous media, such as glucose 5 % or sodium chloride 0.9 %. It is moreover influenced by several parameters such as temperature, concentration and the composition of the container [14, 15]. A possibility to enhance the stability is solution freezing [10, 16].

Previous ceftazidime formulations had extended shelf life stability in a buffered media [6, 17]. However, very few of them used antimicrobial preservative, whereas it is a legal requirement of the European Pharmacopeia (EP) for multidose eye-droppers [18].

The aim of this study was to propose a new ceftazidime multidose eye-drop formulation with a high ceftazidime concentration (50 mg/mL), supplemented with an antimicrobial preservative, namely benzalkonium chloride. Ceftazidime stability was then evaluated as well as preservative efficacy.

Material and methods

Material

All reagents used to produce eye-drops were pharmaceutical grade. They included ceftazidime 2 g (Fortum®, GlaxoSmithKline, Rueil-Malmaison, France), sodium chloride 0.9 % (NaCl 0.9 %) (Ecoflac®, BBraun, Boulogne Billancourt, France), water for injection (Proamp®, Aguettant, Lyon, France), benzalkonium chloride (Inresa, Bartenheim, France), and balanced salt solution (BSS) (Bausch & Lomb, Montpellier, France). An anhydrous pyridine solution was provided by Sigma-Aldrich (Lyon, France).

All analytical reagents were analytical grade, and included potassium dihydrogen phosphate (KH_2PO_4), sodium hydroxide (NaOH), hydrochloric acid (HCl) and hydrogen peroxide solution (H_2O_2) (Sigma-Aldrich). Acetonitrile was provided by Hipersolv Chromanorm (VWR, Fontenay sous Bois, France).

The microorganisms used for antimicrobial preservation efficacy assay were chosen according to EP recommendations: *Staphylococcus aureus* (CIP 4.83), *Pseudomonas aeruginosa* (CIP 82.118) and *Candida albicans* (IP 48.72). Casein soya bean digest agar and Sabouraud-dextrose agar without the addition of antibiotics were supplied ready-to-use (Biomerieux, Marcy l'Etoile, France).

Method

Eye-drops formulation

Ceftazidime powder was reconstituted according to the manufacturer's instructions in water for injection to obtain a concentration of 500 mg/mL. A stock solution of benzalkonium chloride at 0.64 mg/mL concentration in BSS was also prepared. BSS contains sodium (156 mM), potassium (10 mM), calcium (3 mM), magnesium (1 mM), and chloride (129 mM), corresponding to an osmolality of approximately 300 mOsmol/kg, and a pH 7.5.

Stock solutions of ceftazidime (500 mg/mL) and benzalkonium chloride (0.64 mg/mL) were mixed in BSS to obtain a final solution of 50 mg/mL ceftazidime and 0.04 mg/mL benzalkonium chloride. The solution was then sterilized by filtration through a sterile polyethersulfone (PES) filter membrane of 0.22 µm porosity (Stericup®, Merck-Millipore, Darmstadt, Germany). Finally, 10 mL of eye-drops were packaged in sterile-amber-glass eye-droppers (Spruyt Hillen, IJsselstein, Netherlands), corresponding to a brown boro-silicate glass (glass type 1 according to European pharmacopeia). Dropper caps are composed of chlorobutyl-rubber and pigments (titanium dioxide and pigment blau).

Chromatographic conditions

Equipment

A High-Pressure Liquid Chromatography (HPLC) method previously developed by Chédru-Legros *et al.* was adapted [10]. The system was composed of a Serie 200 pump, an injector and an oven with a temperature fixed at 25 °C

(Perkin Elmer, Whatman, USA). A Photodiode Array detector (PDA) operating between 190 to 700 nm completed the system (Flexar PDA detector, Perkin Elmer, Villebon S/Yvette, France). All the equipment was managed by the Chromera software (v4.1.0, Perkin Elmer, Villebon S/Yvette, France). The column was a Supelcosil LC-18 (150 mm x 4.6 mm, 5 µm) (Sigma-Aldrich, Lyon, France).

Conditions

The mobile phase was composed of KH_2PO_4 buffer (0.05 M) with pH adjusted to 2.8 using HCl. It was mixed with acetonitrile (91:9 v/v). The flow rate was set at 1.5 mL/min. The injection volume was 20 µL, the analysis run time was set at 10 min, and detection wavelength was 255 nm. The total area of the peak was used to quantify ceftazidime. Ceftazidime spectra (190–700 nm) were extracted at the apex of chromatogram peaks to detect potential modification of ceftazidime spectra all along the study.

Validation of the HPLC method

The method was validated in accordance with the ICH Q2 R1 [19].

Linearity. Calibration curves from 5 different ceftazidime concentrations ranging from 400 to 600 µg/mL (400, 450, 500, 550 and 600 µg/mL) were prepared in NaCl 0.9 %. Three calibration curves were performed on 3 different days. The mean calibration curve was used to quantify ceftazidime concentrations in further experiments. The r^2 (coefficient of determination) of the mean calibration curve must be greater than 0.995 for the method to be considered linear (Threshold used in the lab as common practice).

Accuracy. Three different solutions of 400, 500 and 600 µg/mL were prepared three times a day for 3 days. Accuracy was determined as the difference between the mean measured value and the accepted true value. Following the current local procedures, accuracy, for each concentration, must be < 5 % from the accepted true value to be accepted.

Precision. Repeatability of the method was evaluated by analysing a 450 µg/mL sample, prepared 6 times a day. Intermediate precision was evaluated by analysing a 450 µg/mL sample prepared 6 times a day for 3 days. Repeatability and intermediate precision were measured using the relative standard deviation (RSD), which must be < 5 % to be accepted.

Stability indicating method. Several stress conditions were applied to ceftazidime. The remaining concentrations were then evaluated using the HPLC-UV method and degradation products were sought thanks to the PDA detector, between 190 and 700 nm. Four different stress conditions were finally applied to a stock solution at 50 mg/mL in NaCl 0.9 %. UV degradation was first carried out over 72 hours. The light degradation was accomplished with an UV-A exposition of 366 nm under a 300 µW/cm² intensity (Chromato-Vue system, model CC-20, Ultra Violet Product, Upland, California). Acid degradation was then assayed by mixing an equivalent volume of the stock solution and 0.1 M HCl. After 90 min, the reaction was stopped and neutralized with an equivalent volume of 0.1 M NaOH. An alkaline degradation was also performed with a 0.001 M NaOH solution. NaOH was mixed to the stock solution (v:v) for 90 min, then the reaction was stopped with an equivalent volume of HCl solution at 0.001 M. Finally, oxidative stress was applied to ceftazidime using a 10 % v:v H_2O_2 solution. H_2O_2 solution was diluted 100 times in water, and an equivalent volume of the diluted solution and ceftazidime stock solution were mixed for 60 min and heated at 60 °C. Pyridine is a well-known degradation product of ceftazidime [20–22]. In this way, a ceftazidime solution, diluted 20.000 times in NaCl 0.9 % was analysed by the HPLC method. A triplicate of each condition was performed.

Stability study

Ceftazidime concentrations

After production, the remaining ceftazidime concentration was evaluated over time to determine the shelf life of the formulation. Different solutions were tested and compared to the initial concentration (just after production), considered as 100 %. Solutions tested included eye-drops stored at 4 °C and eye-drops stored at −20 °C for 75 days then defrosted at room temperature for 30 min (at least, and not more than 2 hours), and stored at 4 °C for the rest of the time (eye-droppers were systematically stored vertically, and droppers were closed). Ceftazidime concentrations were evaluated, on 3 different eye-droppers, for each condition evaluated, using the HPLC-UV method previously described.

Remaining ceftazidime concentration percentages were expressed with a 95 % confidence interval of the mean. The mean and confidence interval were considered acceptable if greater than 95 % of the initial concentration.

Degradation products were systematically explored.

Physical measurements

Osmolality (determined with a vapor pressure osmometer, Vapro® 5520, Elitcechgroup, Puteaux, France) and pH (HI122, Hanna Instruments, Tanneries, France)

of the tested solutions were also recorded. Limpidity of the solution was also explored at every analysis day.

Antimicrobial preservative efficacy

The antimicrobial preservative efficacy of eye-drops formulation was evaluated according to the EP 9.0, part 5.1.3: "efficacy of antimicrobial preservation". Three eye-drops flasks were evaluated.

Frozen *S. aureus* and *P. aeruginosa* strains were plated on Casein soya bean digest agar and incubated at 30 °C for 24 h. Frozen *C. albicans* strain was plated on Sabouraud-dextrose agar and incubated at 25 °C for 48 h. After one subculture, bacterial suspensions were prepared in normal saline (sodium chloride 9 g/L) for each isolate studied. To obtain the suitable microbial concentrations of 10^8 bacteria /mL for bacteria and 10^7 yeast /mL, colony suspension was equivalent to 0.8 McFarland standard (MF) for *S. aureus* and *P. aeruginosa* and to 3 MF for *C. albicans*. The number of colony-forming units per mL was determined by plate count for each microbial strain and this value was used to accurately determine the quantity of micro-organisms inoculated into the eye-drops. For this inoculation, 100 µL of microbial suspension was added to 10 mL eye-drops in order to obtain a 10^6 CFU/mL for bacteria and 10^5 CFU/mL for yeast. Contaminated eye-drops were kept at 25 °C for 28 days. At each time point defined by the EP (T0, 6 H, 24 H, 7, 14 and 28 days), 1 mL eye-drops was removed, and ten-fold serial dilutions were performed in sterile NaCl 0.9 %. 1 mL of undiluted and diluted eye-drops were filtered through a 0.45 µm filter (Merck-Millipore). The filter membranes were then washed three times with 100 mL of sterile NaCl 0.9 %. Finally, filters were placed onto Casein soya bean digest agar or Sabouraud-dextrose agar and incubated at 30 °C or 25 °C for 48 hours depending on microbial strains. The number of viable microorganisms was then determined.

A $\log_{10}$ reduction in terms of viable microorganisms is then observed at defined time points.

Results

HPLC method parameters

The retention time of ceftazidime was 5.6 min, with well-defined and symmetrical peaks (Figure 1(a)). The linearity was determined and the average equation after 3 standard curves was:

$$y = 4 \times 10^{-5} \times + 1.22$$

where x is the area under the curve. The r^2 was greater than 0.995 (0.996).

The accuracy of the method was evaluated on 3 different days (Table 1), as well as the repeatability (Table 2). Intermediate precision was determined using the results obtained during the repeatability determination (mean of the 3 days). All parameters were within the requirements, the method was thus validated to quantify ceftazidime during the study.

Regarding the stability indicating capacity of the HPLC-UV method, to each stress condition tested, a degradation product was detected (Figure 1(c–f)). Moreover, after exposure to degradation conditions, relative ceftazidime concentrations were systematically lower than the initial concentration (Table 3). When a pyridine solution was analysed a peak was detected (Figure 1(g)), with a retention time of 3.0 min.

Physico-chemical stability of ceftazidime eye-drops

Ceftazidime relative concentrations were determined in triplicate at each day of analysis (Table 4). The mean concentration determined immediately after the preparation was considered as 100 %. pH and osmolality were also obtained in triplicate at each time point (Figure 2 (a) and (b)). The 3 parameters demonstrated the stability at 4 °C over 7 days for non-frozen eye-drops and over 75 days for frozen eye-drops. No degradation product was detected in the first 7 days (Figure 1(b)). On day 14 after production (and after defrosting), a peak with a retention time of 3.0 min was detected (Figure 1(c)), suggesting the presence of pyridine in the solution. Moreover, ceftazidime concentration was decreased (Table 4).

Finally, limpidity of solutions was recovered all over time, for all tested conditions.

Antimicrobial preservative efficacy

Before proceeding to assays of antimicrobial preservation efficacy, a validation test was performed for each microbial strain, according to the EP. The number of viable micro-organisms was determined at initial time (immediately after eye-drops inoculation) and compared to the inoculum. As indicated on Table 5, the procedure has permitted to detect the expected amounts of micro-

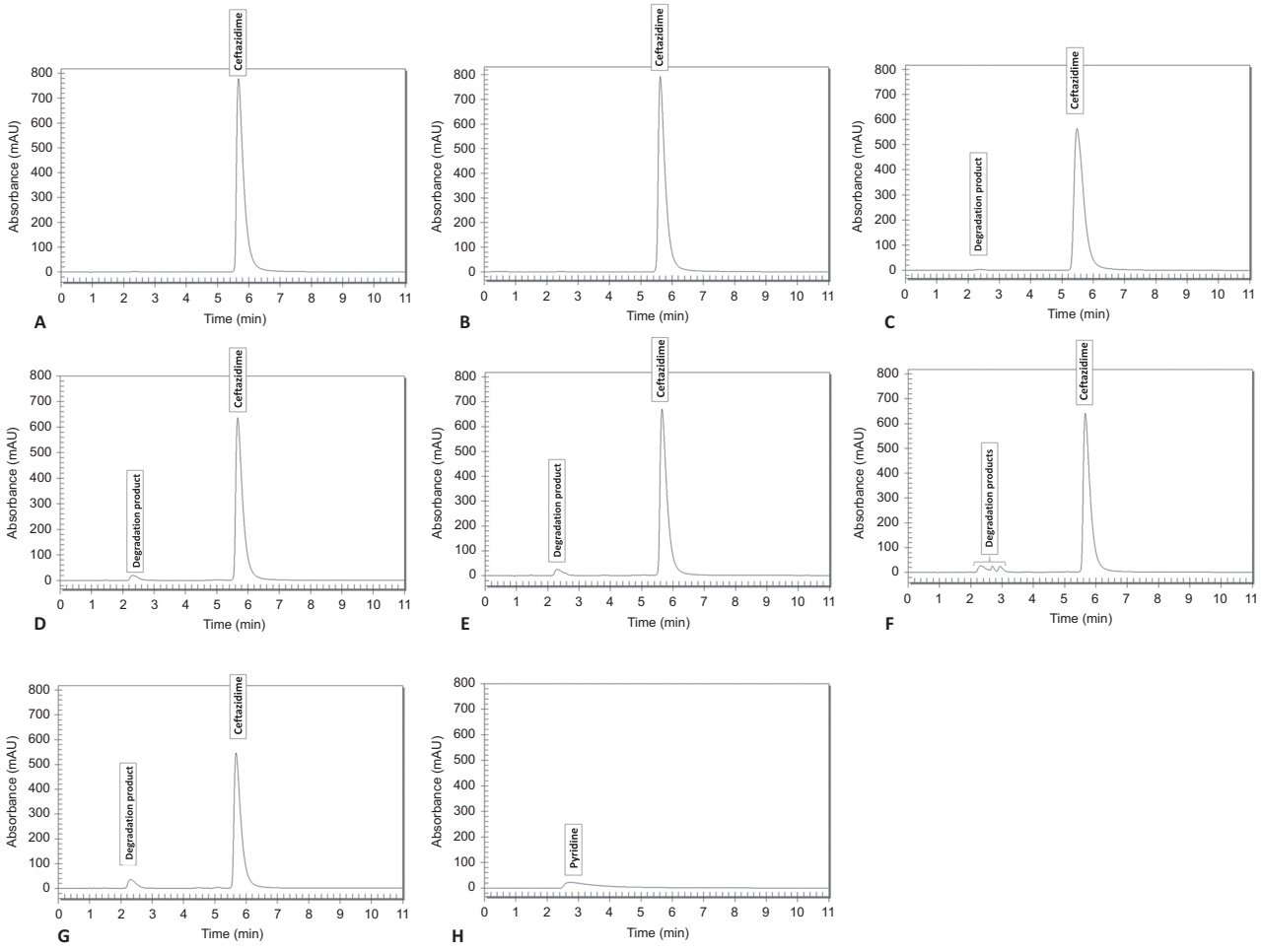


Figure 1: Chromatogram of a 0.5 mg/mL freshly prepared ceftazidime solution (A) analysed by the HPLC -UV method. Chromatograms of a ceftazidime solution stored for 7 days at 4 °C (B) or 14 days at 4 °C (C). Chromatograms of a 0.5 mg/mL ceftazidime solution in contact with an acidic solution (D), an alkaline solution (E), an H_2O_2 solution (F) or exposed to UV (G). A pyridine solution diluted 20,000 times was also analysed (H).

Table 1: Evaluation of the HPLC-UV accuracy. Three different ceftazidime solutions of 400, 500 and 600 µg/mL were prepared three times a day for 3 days. Concentrations are expressed in µg/mL.

Concentration (true value)	Day 1	Day 2	Day 3	Mean ± sd	Accuracy (%)
400.00	444.0	419.6	350.0	416.7 ± 31.5	4.17
	424.4	436.0	407.9		
	428.5	451.8	387.8		
500.00	532.3	527.5	493.1	524.7 ± 16.6	4.94
	531.0	541.4	503.6		
	539.1	535.6	518.6		
600.00	626.0	635.6	602.2	621.7 ± 19.8	3.62
	616.6	651.2	586.6		
	631.3	635.6	610.3		

organisms, indicating that all residual antimicrobial activities of the product have been eliminated by serial dilution, filtration and membrane washing. The procedure has

Table 2: Evaluation of the HPLC-UV method repeatability and intermediate precision. Repeatability of the method was evaluated by analyzing a 450 µg/mL ceftazidime solution, prepared 6 times a day. Intermediate precision was evaluated by analyzing a 450 µg/mL sample prepared 6 times a day for 3 days.

	Concentration (true value) (µg/mL)	Day 1	Day 2	Day 3
Repeatability	450.00	455.1	453.7	489.9
		457.0	492.8	492.3
		470.9	447.4	471.9
		439.6	499.1	521.6
		422.2	439.9	506.2
		474.9	452.2	487.4
	Mean (µg/mL)	453.3	464.2	494.9
	RSD (%)	3.89	4.95	3.15
Intermediate precision	RSD (%)		4.02	

RSD: relative standard deviation.

Table 3: Stress conditions tested to evaluate the stability indicating capability of the HPLC-UV method. Every conditions were performed in triplicate, results are presented as mean ± sd (n = 3).

Stress type	Ceftazidime retention time (min)	Ceftazidime remaining concentration (%)
No degradation	5.64 ± 0.22	100
Acidic degradation (HCl 0.1 M – 1h30)	5.67 ± 0.22	87 ± 6
Alkaline degradation (NaOH 0.001 M – 1h30)	5.55 ± 0.14	83 ± 1
Oxydative degradation (H_2O_2 10 % v:v – 1h – 60 °C)	5.67 ± 0.23	81 ± 2
UV degradation (366nm – 72 hours)	5.67 ± 0.20	73 ± 4

Table 4: Evolution of relative ceftazidime concentrations (in %) over time, expressed as 95 % confidence interval (n = 3). Day 0 corresponds to the first time point of the study and is related to the day of production.

Time points	4–8 °C	4–8 °C (following freezing and storage over 75 days)
Day 0	[99.55–100.45]	[101.02–102.32]
Day 2	[95.60–97.91]	[98.55–99.91]
Day 7	[93.54–96.61]	[99.52–101.18]
Day 14	[86.71–91.56]	[84.64–101.44]

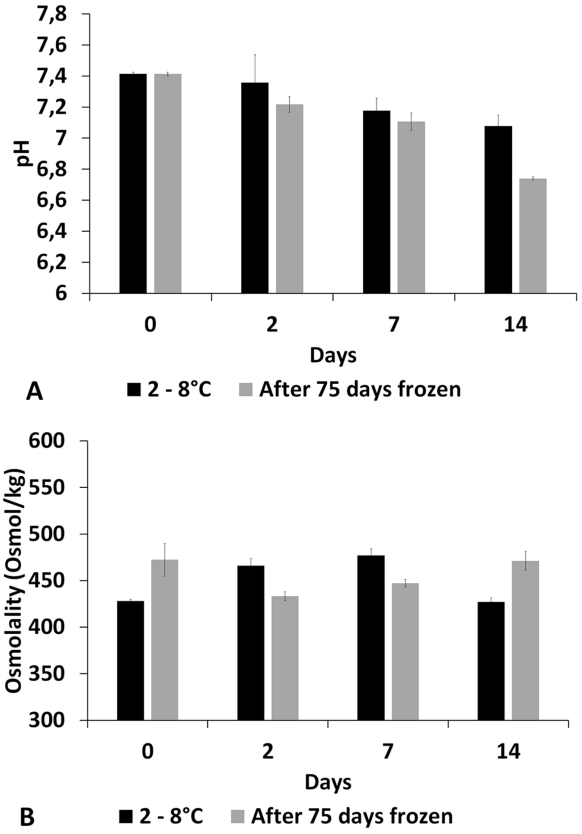


Figure 2: pH (A) and osmolality (B) of ceftazidime eye-drops stored at 4–8 °C for 14 days or ceftazidime eye-drops stored at 4–8 °C after being stored 75 days frozen.

thus been validated and has been applied to examine the reduction of viable micro-organisms.

Efficacy of the antimicrobial preservative (benzalkonium chloride at 0.04 mg/mL) was confirmed (Table 5). For *S. aureus* and *C. albicans* criteria A were obtained, however criteria B was attained for *P. aeruginosa*.

Discussion

Ceftazidime eye-drops are widely used in bacterial keratitis treatment when clinically Gram-negative organisms are suspected as, for example *P. aeruginosa* [10, 23, 24]. Bacterial keratitis requires a rapid antibiotherapy treatment to minimize ocular injury, or in the worst case blindness [25].

A new ceftazidime eye-drops formulation at 50 mg/mL concentration containing an antimicrobial preservative, benzalkonium chloride at 0.04 mg/mL was developed. Glass bottles were chosen due to a previous study showing that ceftazidime residual concentrations were higher in glass bottles after a couple of days than in polypropylene bags or polyvinyl chloride bags. Moreover, pyridine formation, the main degradation product of ceftazidime is also slowed down in glass bottles compared to polypropylene or polyvinyl chloride bags [15]. A new elastomeric device, presenting advantages to glass bottles (i. e. less fragile and more light) for eye-drops is currently being designed to avoid the use of microbial preservative. However sorption phenomena could be observed on silicone used in these devices, and initial concentrations could therefore be decreased [26].

Ceftazidime could be stabilized in a buffered solution [6]. In this way, BSS was chosen to buffer the formulation. BSS has high qualities in ophthalmologic preparations notably thanks to the cations present in the solution, which help to maintain cell homeostasis, which is particularly important for tolerance in the eye [27], as compared to commonly used phosphate buffer. Compared to previously described formulations [6, 10], an antimicrobial preservative was added in the eye-drops as required by EP. This adjunction is performed to reduce microbial contamination

Table 5: Microbial concentration in contaminated eye drops by time points and microbial logarithmic reduction. NR, No Recovery.

		Validation test		Test for efficacy of antimicrobial preservation			
Microbial strains		**inoculum**	**0 hour**	**6 hour**	**24 hour**	**7 days**	**28 days**
Staphylococcus aureus CIP 4.83	log_{10} CFU/mL	6.54	6.15	NR	NR	NR	NR
	Log_{10} reduction	–	–	>6.15	>6.15	>6.15	>6.15
Pseudomonas aeruginosa CIP 82.118	log_{10} CFU/mL	6.78	6.63	5.68	4.15	NR	NR
	Log_{10} reduction	–	–	0.95	2.49	>6.63	>6.63
Cancida albicans IP 48.72	log_{10} CFU/mL	5.08	5.30	–	NR	NR	NR
	Log_{10} reduction	–	–		>5.30	>5.30	>5.30

due to possible contacts between infected eye and eye-droppers, during drop instillation.

Indeed, EP imposes to add a microbial preservative in multidrug delivery eye-drop formulations. Benzalkonium chloride was used, because it is commonly used in eye-drops with marketing authorizations [27, 28]. Nevertheless, it could be toxic in regards to its surfactant properties [27–30]. Its concentration was thus minimized, and a concentration of 0.04 mg/mL was retained. This concentration is commonly found in eye-drops commercially available [27, 28].

Finally, ceftazidime was stable for 7 days at 4 °C for non-frozen and 75 days for frozen eye-drops with remaining concentrations systematically above 95 % of the initial concentrations. It is also stable for 7 days after the freezing period, leading to less dispensations than a previous solution prepared in NaCl 0.9 % [10, 16]. Moreover, osmolality and pH were not different from initial values after a 7-day storage at 4 °C. At day 14, a degradation trend was observed with concentrations below 95 %, degradation products were detected, pH was also slightly different from initial conditions. Osmolalities were very similar to initial conditions at every time. The pH stability for the first 7 days is consistent with a previous study demonstrating that glass bottles do not influence the pH stability of different antibiotic eye-drops solutions [10]. Finally, osmolality is compatible with an ophthalmologic application and the pH is also very close to the physiologic ocular pH, which is a key point to avoid painful reactions during eye-drops application.

The antimicrobial potency of the formulation was assayed on several strains (*S. aureus*, *P. aeruginosa* or *C. albicans*), all recommended by the EP. Antimicrobial efficacy was finally observed confirming the great interest of adding an antimicrobial preservative in formulation, and the minimal concentration of 0.04 mg/mL was considered to be efficient (Table 5).

Conclusion

A ceftazidime eye-drops formulation with a high concentration of ceftazidime was produced. This multidose eye-drops container complies with EP requirements in terms of antimicrobial preservative.

Moreover, a stability was determined for 7 days at 4 °C following a 75-days freezing period, improving patients' quality of life by avoiding return trips to the hospital pharmacy every three days to fill a dispensation.

New formulations with other antibiotics and antimicrobial preservatives are required to be able to propose to clinicians a panel of antibiotics eye-drops in agreements with EP requirements.

Conflict of interest statement: The authors state no conflict of interest. The authors have read the journal's Publication ethics and publication malpractice statement available at the journal's website and hereby confirm that they comply with all its parts applicable to the present scientific work.

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